Resonance Raman spectroscopy of an oxygenated intermediate species of cytochrome oxidase *d* from *Escherichia coli*

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Resonance Raman spectroscopy (excitation at 647.1 nm) of solubilized and aerated cytochrome oxidase *d* from *Escherichia coli* K12 has shown absorptions (1078–1105 cm^{-1}) attributed to the oxygen–oxygen stretching frequency of the oxidase–oxygen adduct. These findings support the hypothesis that the 650–652 nm chromophore of cytochrome *d* is an oxygenated or 'oxy' intermediate species and not the fully oxidized enzyme.

Raman spectroscopy *Oxygen reaction* *Cytochrome oxidase* *Bacterial respiration*

1. INTRODUCTION

Cytochrome oxidase [1] catalyse the reduction of oxygen, generally to water. In the case of the mitochondrial cytochrome *c* oxidase (EC 1.9.3.1), complete reduction to water proceeds via the formation of several enzyme-bound intermediates, which exist only transiently at physiological temperatures. Trapping and spectroscopic characterisation of such intermediates has, therefore, required initiation of the reaction at sub-zero temperatures [2,3] where the activation energies of the constituent reactions favour accumulation of functional intermediates [4]. A striking feature of the cytochrome oxidase *o* of the filamentous myxobacterium *Vitreoscilla* is that an oxygenated intermediate of the enzyme appears to be the predominant steady state form of the cytochrome observable during respiration at physiological temperatures [5]. This has enabled the nature of the bonding of O_2 to the oxidase to be studied using infrared spectroscopy [6].

Under growth conditions where the availability or utilisation of oxygen is restricted [7], *Escherichia coli* synthesises an alternative cytochrome oxidase, cytochrome *d* which has a remarkably high affinity for O_2 with $K_m = 0.024\,\mu M$ [8]. In this organism [9] and in *Azotobacter vinelandii* [10], there is evidence that, in addition to the fully oxidised and fully reduced forms of cytochrome *d*, an intermediate form is observable without recourse to low-temperature trapping. Optical [11,12] and EPR [12] studies of the first compound formed between O_2 and the reduced enzyme at −130 to −140°C suggest that the form of the enzyme absorbing at ~650–652 nm, and generally attributed to the fully oxidised enzyme, is in fact an oxygenated intermediate in the reaction with oxygen.

We report here the resonance Raman spectrum of the 652 nm-absorbing form of cytochrome *d* from *Escherichia coli* and confirm that it is an 'oxygenated' species, analogous in some respects to oxyhemoglobin.

2. EXPERIMENTAL

2.1. *Organism and growth conditions*

Escherichia coli K12 (strain A1002) was grown and harvested extactly as in [13].

2.3. *Preparation of membranes and solubilisation of the oxidase*

Membrane (electron transport) particles were

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prepared by differential centrifugation of an ultrasonically disrupted suspension of cells [14]. Membrane particles (prepared from 19 g wet wt oxygen-limited cells) were suspended at 4°C in 10 ml 50 mM Tris–HCl buffer (pH 7.4) containing 1 mM EDTA, 2 mM $MgCl_2$ and 2% (v/v) Triton X-100. The suspension was sonicated (5 × 30 s bursts at maximum amplitude) and stirred for 30 min at 4°C. The solubilised cytochromes were isolated by centrifugation for 1 h at 200000 × *g*. This procedure rendered 80–85% of the cytochrome *d* soluble. The solubilised cytochrome *d* showed identical spectroscopic properties to those found for the membrane particles. Protein concentration was 2.09 mg/ml and was assayed as in [15].

2.3. *Raman spectra*

Raman spectra were recorded on the University of London Intercollegiate Research Service Spex Ramalog V system at Imperial College (Spex 14018 double monochromator). Sample excitation was achieved with the 647.1 nm emission line of a Coherent Radiation model 52 krypton ion laser. Local overheating was avoided by the use of a spinning cell (2000 rev./min).

3. RESULTS AND DISCUSSION

Attempts were made to record resonance Raman spectra for both the membrane particles and the solubilised cytochrome *d*. In the former case, poor quality spectra were obtained and are not reported here, although certain prominent features were similar to those observed in the spectrum of the solubilised oxidase. The spectrum of cytochrome *d* in the Tris–HCl buffer was recorded under several sets of conditions over 1800–800 cm^{-1} and 500–300 cm^{-1}. The ready precipitation of the solubilised enzyme frustrated the recording of the spectrum over a wider range.

The low frequency spectrum (500–300 cm^{-1}) showed well-defined absorptions at (486(w)), 437(m), 418(s), 392(s), 363(m), 350(w), 343/338(m), 312(w) and 305(w) cm^{-1} (s = strong, m = medium and w = weak intensity), which may be assigned to heme deformation modes and Fe–N (pyrrole) stretching modes [16,17].

The spectrum of cytochrome *d* in the range 1200–800 cm^{-1} showed several absorptions (1183(m), 1155(m), 1085(m,br), 1028(w), 912(w) and 886(w)) (br = broad), the most prominent feature being the broad absorption at about 1085 cm^{-1}. At this stage, the cytochrome *d* was oxygenated to an extent dependent upon the adventitious presence of oxygen in solution. Further oxygenation, accomplished by adding hydrogen peroxide to a sample, or by gently shaking it in an open container, resulted in an increase in intensity in the band at ~1085 cm^{-1} with more pronounced splitting to give maxima at 1105 and 1088 cm^{-1} and a shoulder at 1078 cm^{-1} (fig. 1). Addition of sodium dithionite to reduce the cytochrome *d* resulted in a loss of intensity of these absorptions. On further standing, intensities increas-

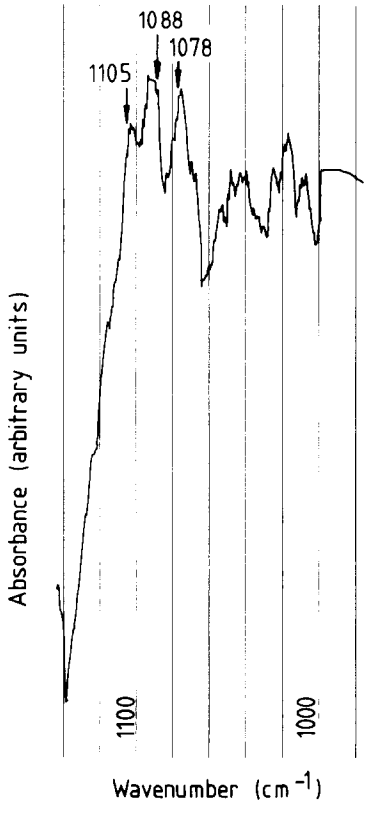

Fig. 1. Resonance Raman spectrum of solubilized cytochrome *d*, oxygenated by the addition of H_2O_2. Excitation was at 647.1 nm; further experimental details are given in the text; protein was 2.1 mg/ml and cytochrome *d* ~1.3 μmol/ml using the absorption coefficient (615–630 nm) of 8.5 $mM^{-1}.cm^{-1}$ [26].

ed as oxygenation re-occurred. Attempts to effect complete reduction by addition of larger amounts of dithionite coincided with precipitation of the enzyme.

The band at ~1088 cm^{-1} may be assigned to the oxygen–oxygen stretching frequency of a cytochrome *d*–oxygen adduct ($\nu(O_2)$). Such frequencies have been assigned for oxygenated cytochrome *o* (1134 cm^{-1}) [6], oxyhemoglobin (1107 cm^{-1}) and oxymyoglobin (1103 cm^{-1}) [18–20] and for a range of oxygenated iron–porphyrin model compounds (e.g., [21]). An oxyhemoglobin study [22] showed three $\nu(O_2)$ bands, at about 1150, 1128 and 1105 cm^{-1}, while mutliple ν(CO) bands have also been observed in the Raman spectrum of carbonmonoxyhemoglobin [23] and carbonmonoxymyoglobin [24]. In contrast, the IR spectrum of oxygenated cytochrome *o* shows only one $\nu(O_2)$ band [6]. It has been suggested that the multiple $\nu(O_2)$ bands in oxyhemoglobin result either from the presence of different conformations or from vibronic coupling of $\nu(O_2)$ with the first overtone of ν(Fe–O) (572 cm^{-1}). We have not yet assigned ν(FE–O) in the case of oxygenated cytochrome *d*. However, it is noteworthy that only one $\nu(O_2)$ band (1072 cm^{-1}) could be observed in the resonance Raman spectrum of cytochrome *d* in the membrane particles. This suggests that the three $\nu(O_2)$ bands in the solution spectrum of oxygenated cytochrome *d* arise from the existence of conformers.

The oxygen–oxygen stretching frequency of the oxygenated cytochrome *d* lies in the region associated with a superoxide group. The precise electronic significance of this is still uncertain, as is reflected in current controversy over the nature of the $Fe(O_2)$ grouping in oxyhemoglobin. However, it is clear that a parallel may be drawn between oxyhemoglobin and oxycytochrome *d*, both with regard to the existence of an oxygenated compound and the very fast recombination of CO after photolysis of the CO compound [25]. This work represents the first use of resonance Raman spectroscopy to describe the mode of oxygen bonding to a cytochrome oxidase, and confirms the view that the 650–652 nm form of cytochrome *d* is an oxygenated intermediate, as first suggested in [9]. A detailed optical and EPR study of the subsequent reactions of this species at sub-zero temperatures, and an assessment of the implications of designating it as an intermediate species will be presented elsewhere.

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